EVALUATION OF METHODS FOR REDUCING PHYTIC ACID IN WHOLE WHEAT DOUGHS

A Thesis

Presented to the

Department of Food Science and Nutrition

Brigham Young University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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December 1984

This Thesis, by Vincient M. Cleamons, is accepted in its present form by the Department of Food Science and Nutrition of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

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ACKNOWLEDGMENTS

This study was supported by a Bennion scholarship and a grant from the Food Science and Nutrition Department at Brigham Young University. Special thanks is given to Dr. Albert E. Purcell and Dr. Kay B. Franz for their guidance, encouragement, and assistance, and to Dr. Clayton S. Huber whose administrative talents and generosity created financial support for my research. My wife, Laurel, and my children, Vincient Jr. and Grace, gave their unconditional love to me throughout this entire project and deserve my deepest gratitude and affection. I am also appreciative of my mother, Barbara Boyd, and my father, the late Gearod Cleamons, who implanted within me the desire to learn and understand.

Finally, I feel it appropriate to acknowledge the help of that supreme power who guides and enlightens all men's minds in their search for knowledge and truth. I am eternally grateful for His influence.

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INTRODUCTION

For centuries phytic acid, a component of whole grains, has posed nutritional problems in areas of the world where these grains are the major food source. With the increased use of whole wheat flour in the United States, there has also been an increased interest in reducing the levels of phytic acid in whole wheat food products.

Phytic acid binds minerals in wheat and other whole grains. The chelation of these minerals decreases their bioavailability and compromises the nutritional status of individuals consuming large amounts of whole grains.

Previous work showed whole wheat doughs adjusted to pH 5.2 had a greater reduction in phytic acid than doughs with a normal pH. Phytic acid was reduced by 70 percent in these doughs after a two-hour incubation period with minimal reduction after four hours. Mayfield found a 7 percent reduction in phytic acid for doughs at pH 5.2 after a five to seven minute period during the

preparation and freezing of the samples. If the destruction was as rapid as indicated, then one would expect a 42 percent reduction in phytic acid after thirty minutes. The purpose of this study is to investigate the effects of pH, time, stirring, and yeast on reducing phytic acid in whole wheat doughs over a two-hour time span.

LITERATURE REVIEW

Phytic Acid

The polyvalent structure of phytic acid contributes to the formation of insoluble salts with polyvalent cations. At the present time the accepted chemical structure of phytic acid is that proposed by Anderson (1914) (Figure 1). The proper name for phytic acid is myo-inositol-1,2,3,4,5,6-hexakis (dilydrogen phosphate) (Cheryan, 1980). Phytic acid is considered the storage form of phosphorus in seeds. It occurs in legumes, grains, and tubers. The reactive phosphate groups of phytic acid can form compounds with monovalent or polyvalent cations and proteins (Barneveld, 1983).

Phytic Acid Salts

Zinc phytate is the least soluble phytate salt, followed by copper, nickel, cobalt, manganese, calcium, and iron, in increasing order of solubility (Cheryan, 1980). Salts formed with monovalent cations are more soluble than the divalent or trivalent cation-salt complexes. The bioavailability of the bound cation increases with increasing solubility of the salt.

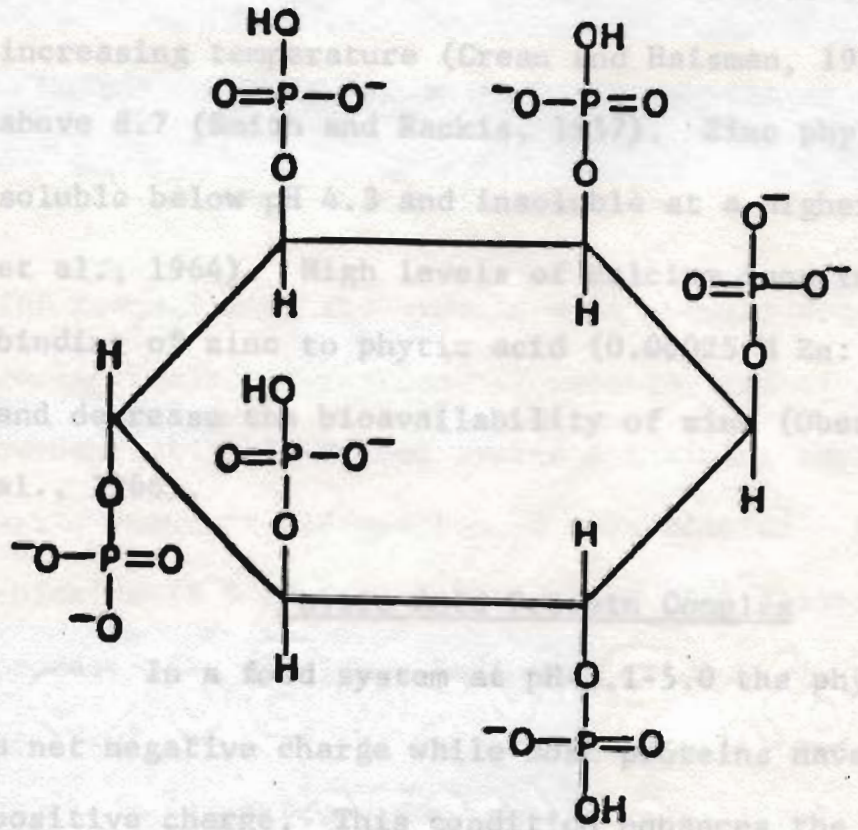


Figure 1. Chemical Structure of Phytic Acid.

Heat, pH, and cation concentrations affect the formation and solubility of the phytic acid salts. Formation of magnesium and calcium salts is increased with increasing temperature (Crean and Haisman, 1963) at pH above 8.7 (Smith and Rackis, 1957). Zinc phytate is soluble below pH 4.3 and insoluble at a higher pH (Allred, et al., 1964). High levels of calcium ions increase the binding of zinc to phytic acid (0.00025 M Zn: 0.03 M Ca) and decrease the bioavailability of zinc (Oberlane, et al., 1966).

Phytic Acid Protein Complex

In a food system at pH 3.1-5.0 the phytic acid has a net negative charge while most proteins have a net positive charge. This condition enhances the binding of free amino groups to phosphate groups of the phytic acid. This complex dissociates at a pH below 3 because phytic acid is less ionized than other anions which combine with the protein.

Both phytic acid and protein have a net negative charge at pH 5-10 as both the phosphate groups of phytic acid and the carboxyl groups of proteins ionize under these conditions. It is postulated that under these conditions the proteins bind to phytic acid via a

multivalent cation. Omosaiye and Cheryan (1979) proposed the following mechanisms for the formation of the salt bridge:

$$\text{Cation} + \text{Phytic Acid} \longleftrightarrow \text{Cation-Phytic Acid Complex}$$

$$\xleftrightarrow{\text{Protein}} \text{Protein-Salt Complex}$$

The formation of the protein salt complex depends upon the concentration of cations. Therefore, reducing the cation concentration in a food system containing phytic acid would reduce the formation of this complex. Sodium chloride (8.5 percent solution) or EDTA dissociates the protein-salt complex. Sodium chloride binds to the carboxyl group of the protein and precipitates the cation-phytic acid residues (deRham and Jost, 1979). EDTA chelates the cation which disrupts the salt bridge.

Phytase

Phytase is a specific phosphatase which catalyzes the hydrolysis of phytic acid to inositol and phosphate. In wheat, phytase exists as two separate fractions, f-1 and f-2 (Lim and Tate, 1973) (Figure 2). Phytase is found in most grains and legumes except soybeans (Cheryan, 1980)

	F-1	F-2
pH optimum[1]	5.6	7.2
Km value	2.2×10^{-5} M	2.0×10^{-4} M
KH_2PO_4 inhibitor		
Ki value (pH 5)	3.0×10^{-4} M	no inhibition at 10^{-2} M
Distribution coefficient (Kd) on Sephadex G-100	0.25	0.25
Apparent molecular weight[2]	47000 + 2000	47000 + 2000
Electrophorectic[3] mobility (Mc) at pH 3.1	0.47	0.58
Lipid cofactor	lysolecithin	not detectable
Optimum temperature	55°	55°

Figure 2. Comparison of Some Properties of f-1 and f-2.

(1) The buffers used for pH determination were 0.5 M sodium acetate for pH 4-5.6 and 0.5 M Tris-HCl for pH 6-8. The enzyme solution was equilibrated with the appropriate buffer before measurement of phytase activity. Km and Ki values were determined from the double-reciprocal plots of reaction rate against substrate (phytate) concentrations. (2) Apparent molecular weights were obtained by measuring the distribution coefficients (Kd) values on sephadex G-100 and interpolation of these points on a standard curve of log molecular weight versus distribution distribution coefficient. (3) Electrophoretic mobilities (Mc) are expressed relative to cytochrome (Lim and Tate, 1973).

The intestinal flora of the rat can synthesize a phytase (Barneveld, 1983) which acts with alkaline phosphatase to hydrolyze phytic acid. Both enzymes are zinc dependent. Davies and Flett (1978) stated:

> . . . if the dietary zinc intake is marginal and the diet contains phytate, the reduction in availability of zinc can lead to a zinc deficiency . . . which decreases the ability of the intestinal mucosa to hydrolyze phytate. One can postulate that the lowered ability of the intestines to metabolize dietary phytate would result in an increased concentration of phytate in the intestinal contents which . . . could bind more zinc and further reduce dietary zinc availability.

Phytic Acid Reduction in Wheat Breads

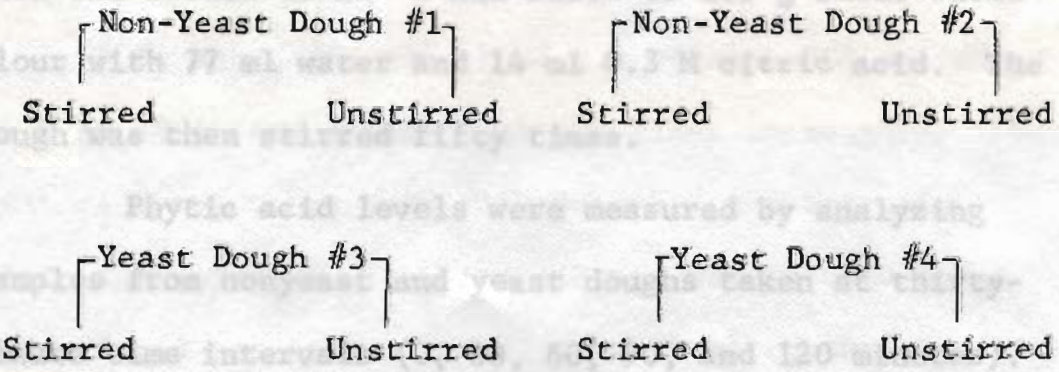
The phytase of wheat causes the breakdown of some phytic acid (Pringle and Moran, 1942). Phosphatases in yeast decrease phytic acid levels in wheat doughs. The addition of yeast and a three to five hour fermentation time further decrease phytic acid in bread doughs (Delange, et al., 1961). Peers (1953) enhanced phytase activity by decreasing the pH of doughs. Mayfield (1983) significantly decreased phytic acid in whole wheat doughs by reducing the pH to 5.2 and allowing the dough to leaven for four hours.

MATERIALS AND METHODS

Experimental Design

The Center for Statistical Research at Brigham Young University outlined the experimental design. The experimental design involved three variables: yeast versus no yeast, stirring versus no stirring, and time of incubation.

To obtain a pH near 5.2, whole wheat flours were mixed with water and citric acid to form viscous doughs. Yeast was added to two of the four doughs. All four doughs were allowed to incubate for two hours with phytic acid levels being measured every thirty minutes. Each dough sample was divided into stirred (stirring after thirty, sixty, and ninety minutes) and unstirred batches.



Dough samples were taken at 0, 30, 60, 90, and 100 minutes. Samples were frozen in liquid nitrogen, then

stored at a temperature below -10° C until being freeze-dried. Freeze-dried samples were then analyzed for phytic acid.

Wheat Dough Preparation

Whole wheat flours (Hansel cultivar) were analyzed and graded by the methods of Mayfield (1983). The flours contained 742 mg phytic acid/100 g flour as analyzed by Mayfield (1983).

Doughs without yeast were prepared using 109 g whole wheat flour, 77 ml water (22° C), and 14 ml 0.3 M citric acid (for dough pH 5.2). After combining these ingredients, the dough was stirred fifty times.

Yeast doughs were prepared by first dissolving 3.4 g of yeast in 4 ml of water at 39.4° C. This yeast mixture was cooled to 22° C and added to 109 g whole wheat flour with 77 ml water and 14 ml 0.3 M citric acid. The dough was then stirred fifty times.

Phytic acid levels were measured by analyzing samples from nonyeast and yeast doughs taken at thirty-minute time intervals (0, 30, 60, 90, and 120 minutes). These samples were immediately frozen in liquid nitrogen (40-60 seconds) and stored at a temperature below -23° C prior to freeze drying.

Some of the nonyeast and yeast doughs were stirred fifteen times after thirty, sixty, and ninety minutes. This was to determine if stirring would increase the interactions between substrate (phytic acid) and enzyme (phytase) to enhance phytic acid hydrolysis.

Phytic Acid Determination

The method for phytic acid determination was described by Mayfield (1983), Ellis and Morris (1982), and Latta and Eskin (1980). The three phases are phytic acid extraction (Figure 3), isolation of sodium phytate (Figure 4), and chelating of sodium phytate by Wade Reagent (Figure 5), after which, the amount of Wade Reagent (0.03 percent $FeCl_3$ - 6 H_2O and 0.3 percent sulfosalicylic acid in distilled water) not bound by phytic acid was measured spectrophotometrically.

In phase one 100 ml of 2.4 percent HCl was added to 5 gm of freeze dried dough and mixed thoroughly. The mixture sat for one hour with intermittant agitation. After one hour, 10 ml of the mixture was removed and centrifuged for five minutes. Then 5 ml of the supernatant was mixed with 5 ml of iron chloride solution (4 g ferric chloride in 1 L of 0.6 percent HCl) and heated for fifteen minutes in boiling water. To ensure a good floc,

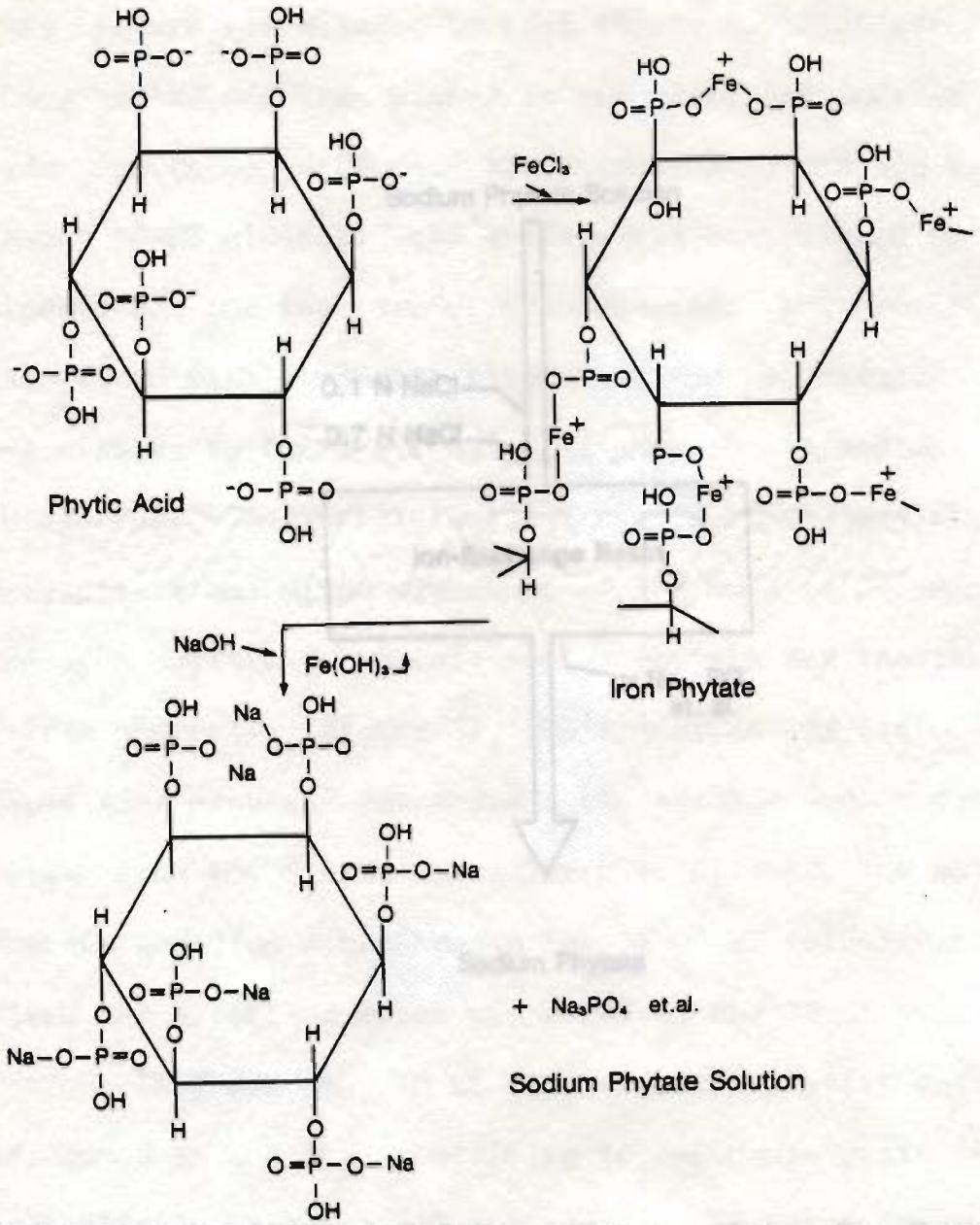


Figure 3. Phytic Acid Extraction

Phase 1: 2-4% HCl was added to freeze dried whole wheat dough to extract the phytic acid. $FeCl_3$ was added to form an iron phytate precipitate. The addition of NaOH decomposed the iron phytate to soluble sodium phytate (sodium phytate solution) and insoluble $Fe(OH)_3$.

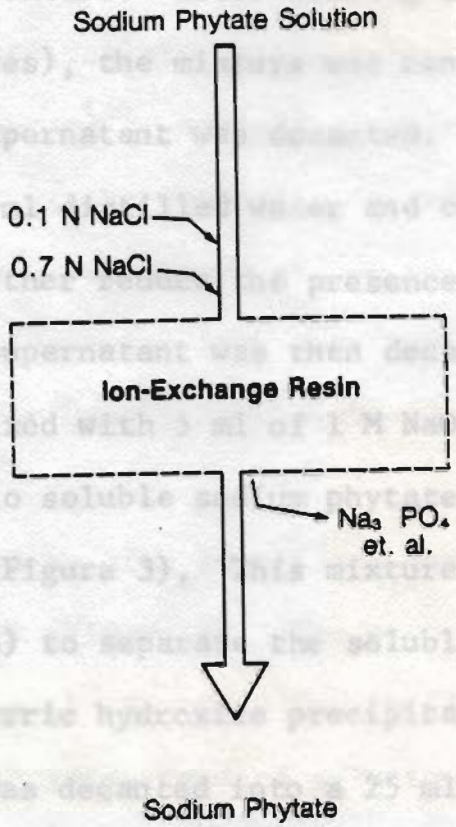


Figure 4. Isolation of Sodium Phytate Using Ion-Exchange Resins.

Phase 2: Sodium phytate solution is added to a column containing ion-exchange resin (0.5 g of 200-400 mesh Dowex 1 x 8 resin). Inorganic phosphates and other interfering constituents were eluted with 0.1 N NaCl. Phytic acid was eluted with 0.7 N NaCl.

this mixture was allowed to cool slowly to 22° C after being heated and then placed in ice water for further reduction in temperature. After cooling in the ice bath (about 10-20 minutes), the mixture was centrifuged for 5-7 minutes and the supernatant was decanted. The precipitate was washed with 5 ml distilled water and centrifuged again, 5-7 minutes to further reduce the presence of sodium phosphates. The supernatant was then decanted and the precipitate was mixed with 5 ml of 1 M NaOH to decompose the iron phytate to soluble sodium phytate and insoluble ferric hydroxide (Figure 3). This mixture was centrifuged (5-7 minutes) to separate the soluble sodium phytate eluant from the ferric hydroxide precipitate. The sodium phytate solution was decanted into a 25 ml volumetric flask and distilled water was added to the 25 ml mark.

In phase two, 10 ml of the sodium phytate solution was added to a column containing ion-exchange resin (0.5 g of 200-400 mesh Dowex 1 x 8 resin). Inorganic phosphates and other interfering constituents were eluted from the column with 15 ml of 0.1 N NaCl. Phytic acid was eluted with 0.7 N NaCl which drained into a 25 ml volumetric flask and was made to the 25 ml mark with distilled water (Figure 4).

Phase three is the formation of the phytic acid-Wade Reagent complex by mixing 6 ml of the sodium phytate solution with 2 ml of Wade Reagent to yield a sodium phytate-iron sulfosalycilate precipitate (Figure 5). The precipitate was removed by centrifuging for ten minutes and the absorbance of the supernatant (unbound sulfosalicyclic acid) was determined at 480 nm on a spectrophotometer (Bauser & Lomb Spectronic 20,6815CW, U.S.A.; Perkin-Elmer Double Beam, 4598-40, Hitachi Ltd., Tokyo, Japan).

The absorbance of a sample was compared with that of a standard curve (Figure 6). The standard curve was made using a series of solutions (ug phytic acid / ml distilled water) to make standard solutions. Distilled water was used as a zero. The standard was run twice during the entire procedure. The absorbance of the Wade Reagent not bound by phytic acid was read. The amount of phytic acid extracted from a particular dough sample was calculated by subtracting the absorbance of the sample from the absorbance of the water blank. This difference was then compared with the absorbance readings of the standard curve, which correspond with various concentrations of phytic acid, to calculate mg phytic acid/100 g whole wheat dough (dry weight).

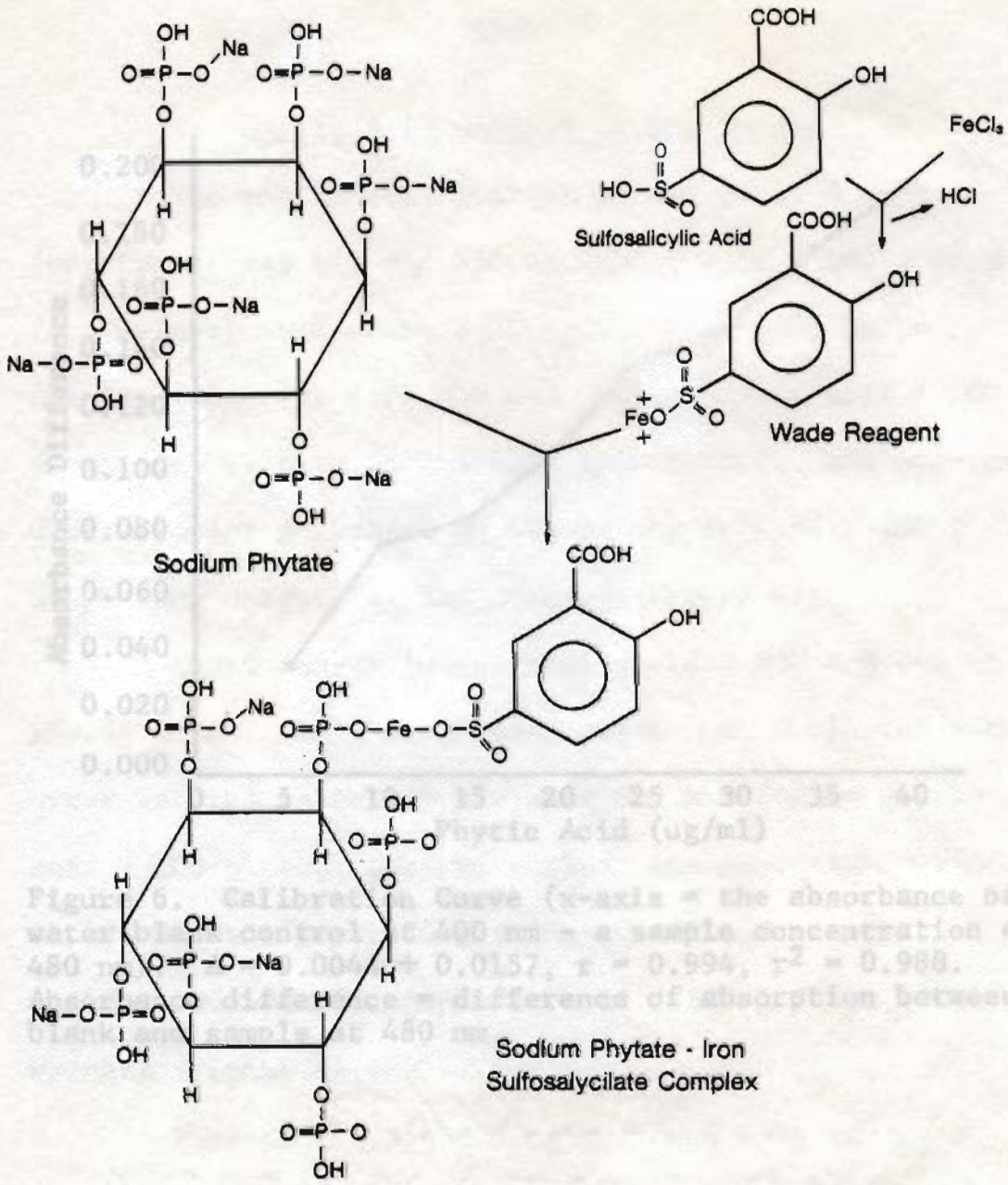


Figure 5. Chelating of Sodium Phytate by Wade Reagent.

Phase 3: Sodium phytate combines with Wade Reagent to form a sodium phytate-iron sulfosalycilate complex.

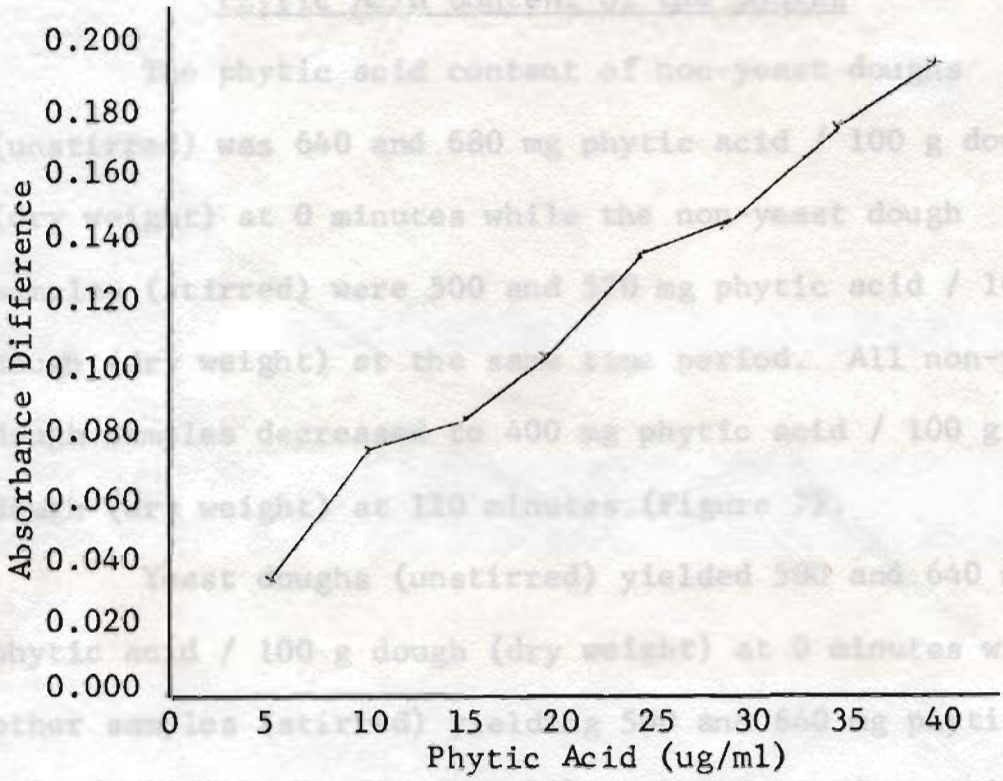


Figure 6. Calibration Curve (x-axis = the absorbance of a water blank control at 400 nm - a sample concentration at 480 nm). A = 0.0044 + 0.0157, r = 0.994, r^2 = 0.988. Absorbance difference = difference of absorption between blank and sample at 480 nm.

RESULTS

Phytic Acid Content of the Doughs

The phytic acid content of non-yeast doughs (unstirred) was 640 and 680 mg phytic acid / 100 g dough (dry weight) at 0 minutes while the non-yeast dough samples (stirred) were 500 and 520 mg phytic acid / 100 g dough (dry weight) at the same time period. All non-yeast dough samples decreased to 400 mg phytic acid / 100 g dough (dry weight) at 120 minutes (Figure 7).

Yeast doughs (unstirred) yielded 500 and 640 mg phytic acid / 100 g dough (dry weight) at 0 minutes with other samples (stirred) yielding 500 and 640 mg phytic acid / 100 g dough (dry weight) at the same time period. At 120 minutes yeast doughs (unstirred and stirred) yielded 400 and 320 mg phytic acid / 100 g dough (dry weight) (Figure 8).

Five of the eight doughs showed no gradual decrease in phytic acid at every thirty-minute time interval. One stirred yeast dough, one stirred non-yeast dough, and one unstirred yeast dough showed increases in phytic acid at 30 minutes (the stirred yeast dough with increased phytic acid at 30 minutes showed the same trend at 90 minutes) (Figure 7). One unstirred nonyeast dough

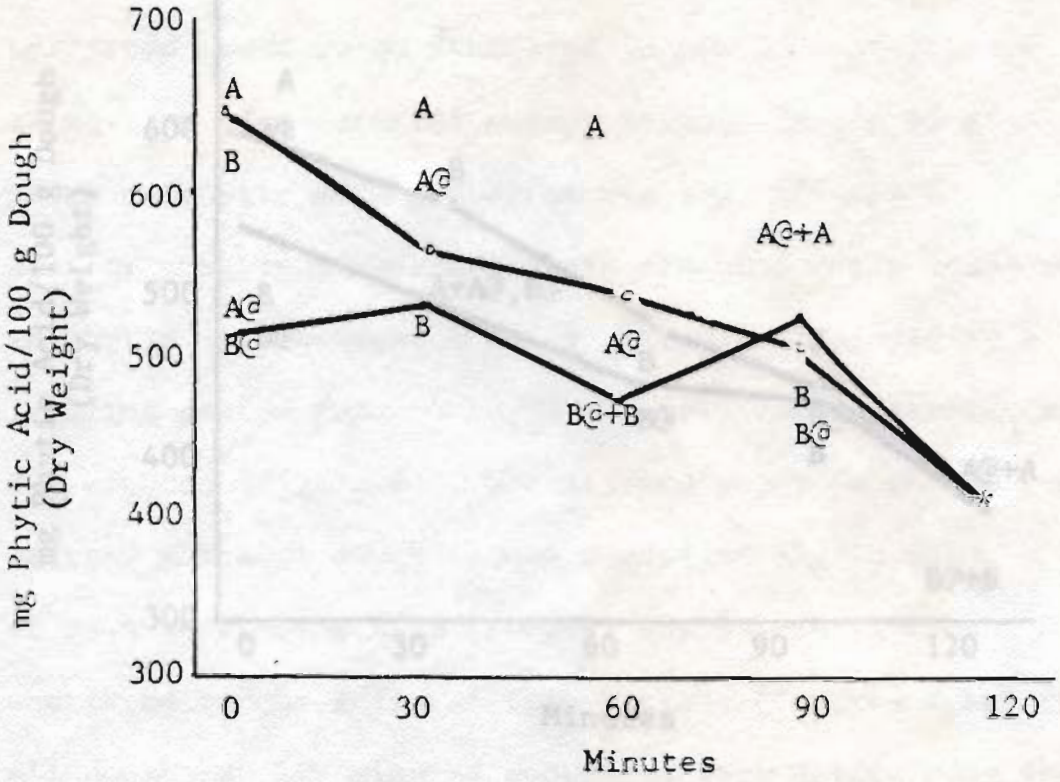


Figure 7. Phytic Acid Extraction from Two Samples of Non-Yeast Doughs (Stirred and Unstirred). Sample A stirred = A@; sample A unstirred = A. Sample B stirred = B@; sample B unstirred = B. Common point of all samples = *. Mean for samples A, B = ·——·. Mean for samples A@, B@ = ——.

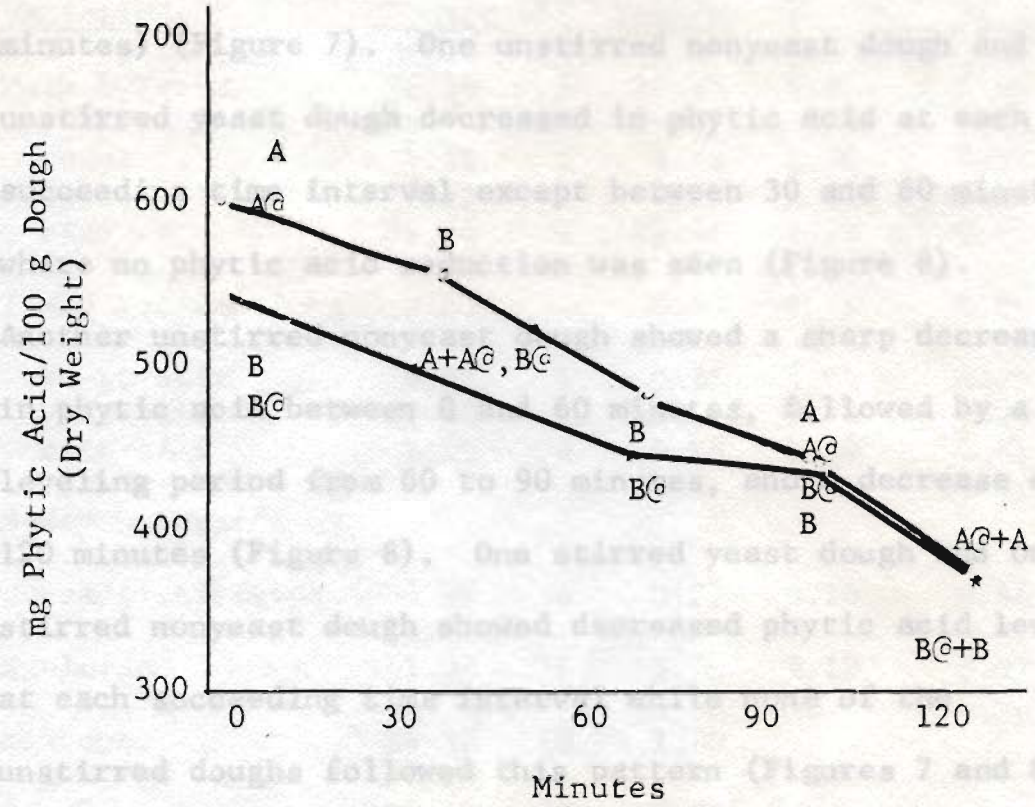


Figure 8. Phytic Acid Extraction from Two Samples of Yeast Yeast Doughs (Stirred and Unstirred). Sample A Stirred = A@; sample A Unstirred = A. Sample B Stirred = B@; Sample B Unstirred = B. Common point of all samples = *. Mean for samples A, B: ◦——◦ . Mean for samples A@, B@:—— .

and one unstirred yeast dough showed increases in phytic acid at 30 minutes (the stirred yeast dough with increased phytic acid at 30 minutes showed the same trend at 90 minutes) (Figure 7). One unstirred nonyeast dough and one unstirred yeast dough decreased in phytic acid at each succeeding time interval except between 30 and 60 minutes where no phytic acid reduction was seen (Figure 8). Another unstirred nonyeast dough showed a sharp decrease in phytic acid between 0 and 60 minutes, followed by a leveling period from 60 to 90 minutes, and a decrease at 120 minutes (Figure 8). One stirred yeast dough and one stirred nonyeast dough showed decreased phytic acid levels at each succeeding time interval while none of the unstirred doughs followed this pattern (Figures 7 and 8). All doughs at 120 minutes contained less phytic acid than that measured at previous time intervals, and significantly less than at 0 minutes.

Statistical analysis shows time to be the only significant variable in reducing phytic acid in whole wheat doughs at pH 5.2 and in a two-hour incubation period (Figure 9). This is based on the F-value of 12.72 (significant if the F-value $>$ 4.35).

Source of Variation	Sum of Square	DF	Mean Square	F	Significance of P
Main Effects	96.18	6	16.03	9.41	0.00
yeast	4.76	1	4.76	2.80	0.11
stir	4.76	1	4.76	2.80	0.11
time	86.66	4	21.66	12.72	0.11
2-Way Interactions	3.83	9	0.43	0.25	0.98
yeast+stir	0.12	1	0.12	0.71	0.79
yeast+time	1.80	4	0.45	0.26	0.90
stir+time	1.90	4	0.48	0.28	0.89
3-Way Interactions					
yeast+stir+time	1.07	4	0.27	0.16	0.96
Explained	101.08	19	5.32	3.12	0.01
Residual	34.07	20	1.70		
Total	135.16	39	3.47		

Figure 9. Statistical Analysis.

DISCUSSION

Phytic Acid Levels during the Two-Hour Incubation

Phytic acid hydrolysis should be greatest in doughs which were stirred and contained yeast. Because stirring should have increased enzyme-substrate (phytase-phytic acid) interaction while the yeast contributes phosphatase. However, the data of Figure 8 did not support this supposition. Therefore, stirring and yeast had little or no effect in reducing phytic acid over a two-hour time period.

Fluctuations in the readings at 30 and 90 minutes may be explained by Ranhotra (1973) and Cheryan (1980) who postulate that the accumulation of phosphates from phytic acid hydrolysed by phytase inhibits the enzyme and leads to rephosphorylation of partially hydrolyzed phytic acid. This same phenomenon was also present in the experimentation by Mayfield (1982). Lim and Tate (1973) isolated two phytase fractions in wheat bran, f-1 and f-2. Fraction f-1 is competitively inhibited by inorganic phosphate, but f-2 is not (Figure 3). It is the f-1 fraction which has a sharp optimum at pH 5.6 while the optimum of f-2 is pH 7.2. The Km values of the two fractions show f-2 to be

ten times more active than f-1, but the concentration of f-1 is three times that of f-2. The temperature of the dough was 18-22° C, which was below that needed for the maximum activity of either enzyme fraction (Figure 2).

Decreasing the diffusion distance of the substrate to the enzyme by additional stirring did not significantly affect phytic acid reduction. Perhaps the stirring was not enough to accomplish this in such a viscous medium.

In view of the report of Lim and Tate (1973), it appears there may have been a greater decrease in phytic acid had the dough been at pH 7.2. However, the work of Mayfield (1983) does not support this. There is also the possibility that the method used was not accurate in measuring phytic acid levels at the specified time intervals or over a two-hour period.

Both enzymes studied by Lim and Tate (1973) have an optimum temperature at 55° C. Suggesting that higher temperatures would increase the rate of reaction and possibly the amount of hydrolysis. If the doughs could be made less viscous, then perhaps phytic acid could be further reduced.

The method for determining phytic acid needs to be improved. Although the method used appears to give more consistent results than other methods, it is least

sensitive at low levels of phytic acid when high sensitivity is most needed. Most spectrophotometers have a logarythmic scale. When reading low levels of phytic acid the absorbance of the sample is near that of the blank and small differences are difficult to detect at the higher end of the absorption scale.

CONCLUSIONS

A two-hour incubation period can significantly reduce phytic acid in whole wheat doughs at pH 5.2. During this time period, phytic acid reduction is independent of yeast (3.4 g dry yeast / 109 g whole wheat flour) and stirring.

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M.S. Degree, August 1984

ABSTRACT

The effects of stirring, yeast, and time on phytic acid in whole wheat doughs at pH 5.2 were explored. Dough samples were prepared and analyzed according to the methods of Mayfield (1982). Phytic acid was reduced significantly in whole wheat doughs at pH 5.2 over a two-hour time period. This reduction was independent of yeast and stirring.

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